THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 64:338–341, 1975

Vol. 64, No. 5
Printed in U.S.A.

THE EFFECT OF ANTHRALIN AND ITS DERIVATIVES ON EPIDERMAL CELL KINETICS

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An attempt was made to clarify the effect of anthralin on epidermal cell kinetics. Commercial anthralin powder was separated by column chromatography into three components: pure anthralin, 1,8-dihydroxyanthroquinone, and anthralin dimer. The effect of these on the diurnal mitotic variation and the cell cycle of the hairless mouse epidermis was studied. Neither anthroquinone nor the anthralin dimer had any influence on these parameters. Both the chromatographically pure and commercial anthralins had similar effects. Mitotic activity was considerably reduced, although not to zero, with concomitant elimination of the diurnal peak. The G_2 and S phases of the cell cycle were approximately doubled in length. Application of the pure and commercial anthralin also resulted in some irritation not seen with either the anthroquinone or dimer.

The first use of anthralin (1,8,9-trihydroxyanthracene) for topical therapy of psoriasis was derived from a report on chrysarobin, a related compound, in 1878 [1]. In treating psoriatic lesions with commercial anthralin, two side effects have been documented: staining of skin and clothing, and irritation of surrounding normal skin. This report is, in part, directed toward determining whether the therapeutic effect and side effects of commercial anthralin are caused by the pure anthralin or a contaminant. Ponec-Waelsch and Hulsebosch [2] have suggested that anthralin oxidation occurs in the presence of an adsorbing surface in a moist environment, resulting in a pink to violet discoloration and an ineffective preparation. Whitefield [3] indicated that oxidation immediately before or after application to the skin could result, respectively, in irritation and staining during anthroquinone formation.

Psoriasis is a skin disease characterized in part by epidermal hyperplasia and which should therefore be controllable with antimetabolic drugs. It has been assumed that anthralin is acting in this manner, i.e., by blocking cell division, but little work has been done to confirm this. Swanbeck and his collaborators [4,5] have indicated that anthralin inhibits DNA synthesis in guinea-pig epidermis and this may be due to binding with the DNA molecule. However, this does not correlate with our inability to demonstrate antimitotic action on normal human skin [6].

The effect on psoriatic epidermis is also in dispute. Baxter and Stoughton [7] demonstrated a significant decrease in the mitotic activity. Fry and McMinn [8] showed reformation of the granular layer after 7 days, but no significant fall in mitosis until after 3 weeks of treatment; while Born [9] was unable to show any effect on DNA synthesis for up to 5 weeks after initiating treatment. Steigleder et al [10] and Pullman et al [11] have measured the epidermal cell kinetics of psoriatic lesions in vitro and indicate that DNA synthesis is slower than normal, i.e., S is longer for psoriatic cells than normal cells. Steigleder et al [10] also show that anthralin returns the kinetics to normal in their system, i.e., anthralin would be speeding up DNA synthesis in psoriatic cells. However, irritation is a possible source of error since this has been shown to cause an increase in mitotic activity of the epidermis [12; Fisher, unpublished data]. In the following work, the known anthroquinone and dimer [13] were separated from pure anthralin in the commercial product to determine which of these may be responsible for any effect on the epidermal cell cycle.

MATERIALS AND METHODS

Purified anthralin, anthroquinone, and anthralin dimer were separated from commercial crude anthralin by silica gel column chromatography. Silica gel G was suspended in heptane and poured into a 1-inch column so that the settled height was 4 inches. The column was then equilibrated with a mixture of benzene, chloroform, and heptane (1:1:1). One hundred milligrams of crude anthralin dissolved in 2 ml of chloroform was placed on the column and the fractions were separated using the chloroform–benzene–heptane mixture. The pale, yellow-colored anthralin moved most rapidly, followed by the orange anthroquinone and deeper yellow dimer. A brown-violet colored contaminant remained at the top of the

Manuscript received August 28, 1974; in revised form November 21, 1974; accepted for publication November 25, 1974.

This work was presented at the 35th Annual Meeting of The Society for Investigative Dermatology, Inc., June 21–23, Chicago, Illinois.

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column. When the pure anthralin layer reached the end of the column, the column was dismantled, the various gel bands removed, and the compounds were eluted from the gel with heptane. The various eluates were dried under nitrogen and resuspended in ethyl acetate. The purified compounds were identified by their absorption spectra and by thin-layer chromatography according to the method of Segal et al [13].

Adult male hairless mice, age 3 to 4 months, were used in this study. All animals were injected with 30 μc of tritiated thymidine (27.0 mCi/mM) at 6:00 AM ± 15 min. One group of 39 animals had no further treatment, while 24 μg of commercial anthralin in 0.1 ml of ethyl acetate (EtAc) was pipetted onto the backs of a second group immediately after thymidine injection. In a subsequent experiment (later date, same time of day), groups of 27 animals were treated with the same quantities of either purified anthralin, anthralin dimer, or anthroquinone. At this time, two groups of 18 animals were also used to determine the effect of 0.1 ml EtAc alone and to confirm the effect of commercial (nonpurified) anthralin.

Immediately after treatment 3 animals from each group were killed by neck pulling. This was repeated at varying intervals (see Figures for specific times). Specimens of skin were removed from the treated (lumbar) regions of the back and fixed in Bouin's solution. Standard techniques were used for wax embedding the specimens. Sections were then cut at 4 μ, dipped in Kodak NTB-2 emulsion, and maintained in a light-tight box containing "Drierite" at 4°C for 21 days. Subsequently, the slides were developed and then stained with hematoxylin and eosin. From these specimens the mitotic index (mitoses per thousand viable epidermal cells [14]) and the percent labeled mitoses were counted using a hand tally counter. A minimum of 50 labeled mitoses were counted from each animal. The mitotic index was plotted against the time of day, and to determine the cell cycles, percent labeled mitoses were plotted against time after ^{3}H-TdR injection.

RESULTS

A review of the histology of the animals showed that the doubling of epidermal thickness, probably indicative of irritation, occurred with both the commercial and the purified anthralin beyond 24 hr after application. This was not observed with the quinone and the dimer. Gross observation of the skin showed slight orange-yellow staining with the pure anthralin and marked orange staining with the quinone. The chromatographically purified compounds were stable in vitro extending beyond the period of these experiments, although no statement can be made with regard to the situation on the animals' skins. However, over a period of several weeks in the test tube, the purified anthralin, anthroquinone, and anthralin dimer converted to a brown-violet compound although this color staining was not observed on the mice during the experimental period.

Diurnal Mitotic Activity

Those animals which had been treated only with ethyl acetate showed no difference from the typical diurnal mitotic variation normally seen in untreated animals (Fig. 1*a*), i.e., a peak mitotic activity was found at 24-hr intervals occurring at approximately 8:00 AM. Animals whose skins had been treated with either the commercial anthralin or the pure anthralin showed a profound inhibition at 4 hr followed by some variability about the control values for the next 14 hr (Fig. 1*a*). However, the expected peak, seen in the control animals at around 8:00 AM, did not occur. Mitotic activity fell to a minimum at approximately 10 hr after drug application and no significant recovery from this value was seen for the 34-hr duration of the experiment. This difference was significant at $p <$

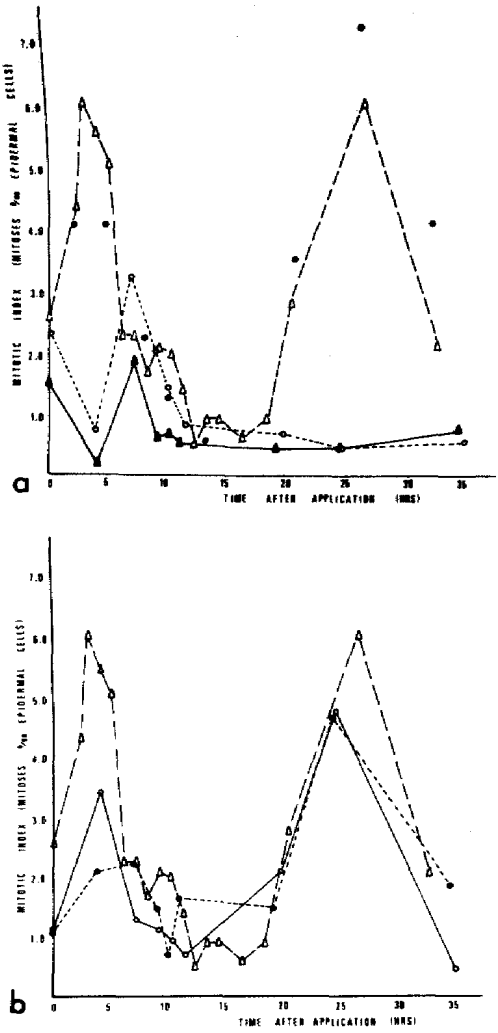

FIG. 1. The diurnal mitotic cycle of hairless mouse epidermis. *a*. Δ— —Δ, Untreated animals; ●, treatment with 0.1 ml ethyl acetate (EtAc); O-----O, treatment with 0.1 ml purified anthralin/EtAc; ▲——▲, treatment with 0.1 ml commercial anthralin/EtAc. All treatment applications at time 0. *b*. Δ— —Δ, Untreated animals; O——O, treatment with 0.1 ml anthroquinone/EtAc; ●-----●, treatment with 0.1 anthralin dimer/EtAc.

.01 level. Those animals which had been treated with the quinone and also the dimer showed no significant difference as compared with the control groups (Fig. 1*b*) except at 4 hr where some mitotic inhibition was seen.

Cell Cycle Analysis

Those animals whose skins had been treated with ethyl acetate alone (Fig. 2*a*) showed no difference in cell cycle analysis from that of the untreated controls. The duration of the S phase as calculated from the 37.5% level of the peak was approximately 6 hr. Animals treated with either the commercial or the purified anthralin, however, showed an S phase increased to approximately 12 hr (Fig. 2*a*). The peak of labeled mitoses was also delayed as compared with the control. This suggested an increase in the G_2 duration from 3.5 hr to 6 to 9 hr. However, the anthroquinone and anthralin dimer caused no change in cell cycle kinetics as compared to the untreated animals (Fig. 2*b*). Since the data could be interpreted to indicate two effects—some cells being completely blocked and others only slowed in their passage through the cell cycle—it is possible that, for some reason, the latter received less anthralin than the former. An attempt was made to reduce mitotic activity to zero by applying anthralin 3 times at 3-hr intervals starting, as in earlier experiments, at 6:00 AM. Animals were killed at 3:00 PM, i.e., 12 hr after the first application. The results in the Table show that this treatment did not produce any greater inhibition than a single application. In fact, the mitotic activity was higher after 3 applications, which could possibly be explained on the grounds of irritation.

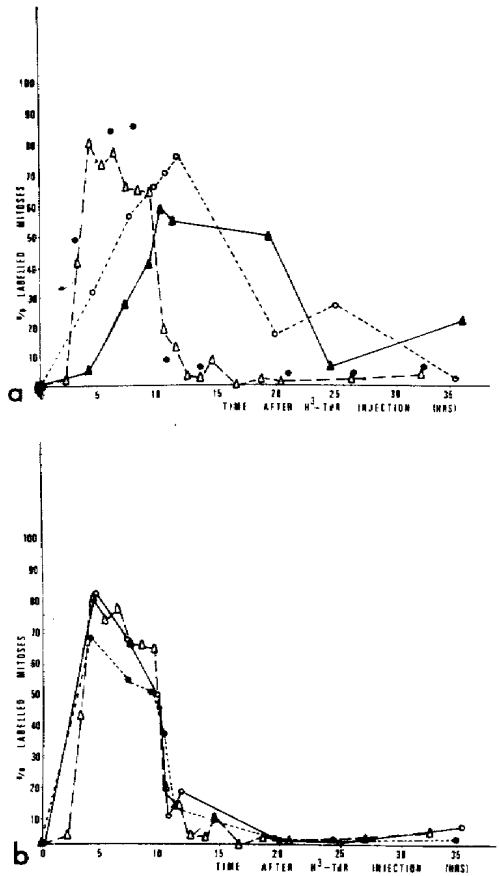

FIG. 2. The cell cycle analysis curve of hairless mouse epidermis. *a*. Δ— —Δ, Untreated animals; ●, treatment with 0.1 ml EtAc; O-----O, treatment with 0.1 ml purified anthralin/EtAc; ▲——▲, treatment with 0.1 ml commercial anthralin/EtAc. *b*. Δ— —Δ, Untreated animals; O——O, treatment with 0.1 ml anthroquinone/EtAc; ●-----●, treatment with 0.1 ml anthralin dimer/EtAc. All treatment applications at time 0.

DISCUSSION

Since an approximate doubling of epidermal thickness was seen after anthralin treatment, this might have artificially produced a decrease in mitotic activity. It is, however, unlikely since the hyperplasia was not apparent until about 24 hr after treatment, at which time the fall in mitosis was well established. With the protocol used in this experiment, any alteration of epidermal cell kinetics appears due to the effect of the anthralin as such, and not to either of the two "impurities" found in the commercial product. Apparently epidermal cell production is slowed in two ways: Firstly, both the S and G_2 phases of the cell cycle are lengthened and this could explain the very early fall in mitotic index seen in Figure 1*a*. Secondly, the cells producing the diurnal mitotic peak are prevented from dividing by a single anthralin application. The data in the Table indicate that anthralin is acting as a complete cell cycle block on one portion of the proliferative pool while only slowing the remainder during G_2 and S.

TABLE. *The effect of multiple anthralin applications on the mitotic activity of hairless mouse epidermis*

Untreated control	Single[a] application	Multiple[b] applications
1.06	1.08	1.52
3.57	0.44	1.70
1.85	1.17	1.32
2.32	0.83	1.20
1.00	0.92	1.02
1.66	0.81	1.49
Mean ± S.E. 1.91 ± 0.39	0.88 ± 0.10	1.38 ± 0.10

[a] 24 μg anthralin in 0.1 ml EtAc applied at 6:00 AM.

[b] Three applications of anthralin at 6:00 AM, 9:00 AM, and 12:00 noon 0.1 ml EtAc applied at 6:00 AM to controls.

Control vs 1 application $p < 0.05$.

Control vs 3 applications $p = 0.05$.

These results could also explain why little or no effect was seen in our earlier treatment of normal human skin with anthralin [6]. Although anthralin does indeed depress mitotic activity, the minimum seen after treatment does not fall significantly below the normal daily minimum. The time at which specimens were taken in our treatment of human skin was at approximately 3:00 PM, which is the normal daily minimum for human skin. It is thus possible that our finding in this instance was due to the use of inappropriate timing. This work will have to be pursued on human material for confirmation. Preliminary (unpublished) data indicate that anthralin may be having a similar effect on psoriatic lesions, i.e., there is mitotic inhibition at 8:00 AM but not at 4:00 PM after prior application of the drug in ethyl acetate.

Any effect on epidermal cell kinetics, and therefore possibly any therapeutic effect in psoriasis, is due to anthralin and not to either the quinone or dimer which are also found in the commercial product. Also the oxidation products of anthralin, which showed no biologic activity in the system described here, are much more intensely colored than the purified drug. This provides a reasonable explanation for the finding of Comaish et al [15] that discolored anthralin pastes (possibly containing large amounts of the quinone) are ineffective in treating psoriasis.

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